Rejection of Claim 18 under 35 U.S.C. §101

The Examiner states that claim 18 is rejected under 35 U.S.C. §101 "since the claimed invention is not supported by either a credible asserted utility or a well established utility for reducing the number or activity of macrophages in culture, other than for research purposes."

Applicants respectfully traverse the Examiner's position that the subject matter of claim 18 lacks a patentable utility.

Claim 18 is drawn to a method of selectively reducing the number or activity of macrophages in culture, by contacting the macrophages with a macrophage-binding compound having an agent which binds to an Fc receptor and an agent which kills or reduces the activity of the macrophages.

As described, e.g., at page 37, of the specification, the method of claim 18 has "several diagnostic, therapeutic and research utilities" resulting from the fact that the claimed macrophage-specific agent can be "administered to cells in vitro (in culture), ex vivo [which would also be in culture]...to treat, diagnose or study a variety of disorders." The specification further teaches that the "[m]acrophage-binding compounds can be initially tested in vitro" and provides protocols for establishing macrophage cultures from subjects (page 37, line 29 to page 38, line 8).

In addition, the specification teaches that the methods of the invention can be used for treating various diseases involving, for example, open wounds or burn wounds. Accordingly, based on this, the claimed method is asserted to be useful in, for example, reducing or eliminating the number of macrophages in a tissue sample for transplanting to a recipient subject having such a wound so as to minimize the risk of an undesired immune response. As evidenced by the publication by Taylor *et al.* (*Cryobiology* 24(2):91-102 (1987) (enclosed as Appendix B), in vitro methods for reducing the number or activity of macrophages in a tissue graft prior to transplant was a utility recognized in the art at the time of the invention. Accordingly, because the method of claim 18 accomplishes this useful result, the claimed method has a patentable utility.

Applicants respectfully note that all that is required to meet the requirements of 35 U.S.C. §101 is a ***single utility***. Moreover, the PTO Utility Guidelines provide that inventions having ***well-established*** utilities comply with the utility standard under 35 U.S.C. §101, ***regardless of whether Applicants have explicitly disclosed these utilities within the four corners of the specification***. As defined in the Utility Guidelines, the definition for well-established utility is as follows:

> An invention has a well-established utility ***if a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention*** (e.g., properties of a product or obvious application of a process). (P.T.O. Utility Guidelines, 60 Fed. Reg. 36: 263-302 (July 14, 1995), emphasis added).

That well-established utilities (even if not disclosed in the specification) comply with the utility standard under 35 U.S.C. §101 is also stated in the Supplementary Information to the PTO Utility Guidelines 1170 TMOG 482-489 as follows:

> Occasionally, an applicant will not explicitly state in the specification or otherwise assert a specific utility for the claimed invention. In such cases, ***if a person of ordinary skill would recognize a utility for the claimed invention if provided with the specification at the time of its filing, no rejection under §101 should be imposed***. For example, if an application teaches the cloning and characterization of the nucleotide sequence of a well-known protein such as insulin, and those skilled in the art at the time of filing knew that insulin had a well-established use, it would be improper to reject the claimed invention as lacking utility under §101. (emphasis added)

These guidelines are clearly met in the instant case. Not only do Applicants assert in the specification several credible utilities for selectively reducing the number or activity of macrophages in culture (e.g., in a tissue sample prior transplantation), but these and other utilities also were known in the art at the time of the invention.

Accordingly, Applicants respectfully request that the rejection of claim 18 under §101, be reconsidered and withdrawn.

Rejection of Claim 18 Under 35 U.S.C. § 112, First Paragraph

The Examiner also rejects claim 18 under §112, first paragraph because, based on the lack of utility for claim 18 asserted in the previous subsection, one skilled in the art would not know how to use the invention. In particular, the Examiner states that "since the claimed invention is not supported by either a substantial asserted utility or a well established utility…one skilled in the art clearly would not know how to use the claimed invention."

In response, Applicants reiterate the foregoing arguments which point out that, contrary to what the Examiner asserts, claim 18 is supported by both a credible asserted

utility and a well established utility (see Appendix B). Thus, the rejection is believed to be moot. Based on Appicants' description and the knowledge in the art at the time of the invention, one of ordinary skill could practice the method of claim 18 without undue experimentation.

Accordingly, Applicants respectfully request that the rejection of claim 18 under §112, first paragraph, be reconsidered and withdrawn.

Rejection of Claims 2-6, 8-12, and 19-21 Under 35 U.S.C. § 112, First Paragraph

The Examiner rejects claims 2-6, 8-12, and 19-21 under 35 U.S.C. §112, first paragraph, as "containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to...make and/or use the invention. Specifically, the Examiner states that the specification is not enabling "regarding the prevention of said disease" without undue experimentation (emphasis in the original).

Applicants respectfully traverse this rejection. The specification fully enables the selective reduction of the number or activity of macrophages in a subject using the methods of the present invention. This result can be applied both to prevent and to treat diseases. Thus, the subject matter of claims 2-6, 8-12, and 19-21 is fully enabled.

Moreover, enablement is judged based on the knowledge and level of skill in the art at the time of the invention. At the time of the present invention, the knowledge and level of skill in the art for extrapolating from in vivo animal model data provided in Applicants disclosure, combined with the guidance provided within the four corners of Applicants' specification, would have enabled the skilled artisan to have practiced the present invention as currently claimed without undue experimentation.

Applicants' disclosure provides a detailed description of in vivo animal results (e.g., in Example 9), where an animal model having a chronic skin disease is prevented from showing any evidence of disease for a prolonged period of time when the methods of the invention are applied to the test animal. Moreover, Applicants provide in the subsection entitled "Pharmaceutical Compositions and Administration Routes" (pages 28-37) extensive detail on how to extrapolate from such experimental data, formulations and dosages, both to prevent and to treat macrophage-mediated diseases in humans. Since manifestation of disease states characterized by aberrant macrophage activity or numbers is clearly arrested by applicants claimed method, as evidenced by this Example, a method for "treating or preventing a disease characterized by aberrant activity or number of macrophages is clearly enabled.

Overall, Applicants' specification provides unequivocal enabling support in the form of both written description and working examples for "treating or prophylactically preventing disorders characterized by aberrant numbers or activity of macrophages" and for "preventing or delaying the occurrence of the onset or recurrence of a macrophage-mediated disease state" using the claimed methods (page 40, lines 1-4, 21-24). Provided with this information, including in vivo examples, the skilled artisan could readily apply the claimed methods as a prophylaxis for preventing disease without undue experimentation. Moreover, the Examiner has not provides any reason why one of ordinary skill would doubt such enablement (i.e., that the specification enables prevention, in addition to treatment, of disorders characterized by aberrant numbers or activity of macrophages as claimed).

Thus, by providing the above-described techniques and in vivo data for administering a therapy for preventing a disease characterized by aberrant activity or number of macrophages, Applicants' disclosure meets the requirements of 35 U.S.C. § 112, first paragraph. Accordingly, it is respectfully requested that the rejection be reconsidered and withdrawn.

Rejection of Claims 1-6 Under 35 U.S.C. § 102 (a)

The Examiner rejects claims 1-6 under 35 U.S.C. §102 (a) as being anticipated by Curnow, R. (*Cancer Immunol. Immunother.* 45:210-215 (1997)) as evidenced by Graziano et al. (*J. Immunol.* 155:4996-5002 (1995)). In particular, the Examiner alleges that Curnow, R.T. teaches that mabH22 "binds circulating monocytes causing monocytopenia and down modulates CD64…indicat[ing] its importance in the treatment of autoimmune disorders," and that the agent disclosed by Graziano has the function of the claimed invention.

Applicants respectfully traverse this rejection.

As amended, claims 1-6 are drawn to a method of selectively reducing the number or activity of macrophages (e.g., to treat a disorder characterized by aberrant numbers or activity of macrophages) using a combination of an agent which specifically binds to macrophages via an Fc receptor and an agent which kills or reduces the activity of the macrophages.

In contrast, Curnow teaches that mabH22 (i.e., MDX-33) binds to circulating monocytes causing monocytopenia and down modulation of CD64 (FcγRI) and the potential for using such an agent in the treatment of monocyte-mediated disorders, such as ITP. Curnow fails to teach any method of using H22 to reduce the number or activity of macrophages, as claimed by Applicants, much less specifically to treat or prevent macrophage-mediated disorders, as claimed by Applicants. Although monocyte-derived,

macrophages are well known in the art to be phenotypically and functionally distinct from monocytes. For example, macrophages generally are not freely circulating as are monocytes. Therefore, Curnow fails to anticipate the presently claimed invention. Graziano et al. is cited merely as evidence that H22, as taught by Curnow, binds the FcγRI receptor outside the natural ligand binding site and can be used to treat human diseases. However, this reference fails to teach or suggest the specific use of H22 to treat macrophage-mediated disorders by selectively reducing the number or function of macrophages, let alone in combination with other agents which kill or reduce the activity of macrophages as claimed by Applicants.

Accordingly, it is respectfully requested that the rejection under 35 U.S.C. § 102 (a) be reconsidered and withdrawn.

Rejection of Claims 1-6 and 18 Under 35 U.S.C. § 102 (b)

The Examiner rejects claims 1-6 and 18 under 35 U.S.C. §102 (b) as being anticipated by Erickson et al. (*British Journal of Haematology*, 92:718-724 (1996)). Erickson allegedly teaches a monoclonal antibody that binds monocyte-like cells and is capable of FcγRI internalization activity. Specifically, the Examiner states that the rejection is made on the basis that the monoclonal antibody of Erickson (i.e., mab197) has the function of the agents recited in the claims thereby anticipating the claimed invention.

Applicants respectfully traverse this rejection. As pointed out above, the claims are drawn to a method of reducing the number or activity of macrophages (e.g., to treat or prevent diseases associated with an aberrant activity or number of macrophages), by contacting or administering an agent which binds to an Fc receptor and an agent which kills or reduces the activity of the macrophages.

Like Curnow, the teachings of Erickson relate only to peripheral blood mononuclear cells (i.e., circulating monocytes). Erickson fail to teach or suggest a method of treating macrophage-mediated disorders, as claimed by Applicants. Moreover, the method taught by Erickson does not employ an agent which binds to an Fc receptor at a site which is distinct from that bound by endogenous immunoglobulins, as required by claim 1. Accordingly, the method of claims 1-6 and 18 is not anticipated by this reference.

Accordingly, it is respectfully requested that the rejection under 35 U.S.C. § 102(b) be reconsidered and withdrawn.

Rejection of Claims 1-2, 8-12, and 21 Under 35 U.S.C. § 103(a)

The Examiner rejects claims 1-2, 8-12, and 21 under 35 U.S.C. §103(a) as being unpatentable over Curnow, R.T. (cited *supra*), Graziano et al. (cited *supra*), Erickson et al (cited *supra*), Uhr et al. (USPN 5,686,072), Ghetie et al. (USPN 5,578,706), Rybak et al. (USPN 5,840,840), Pastan (USPN 5,489,525), and Bjerke et al. (ACTA Derm. Venereol. (Stockh) Suppl. 186:141-142 (1994)).

In particular, the Examiner states that in combination, the references of Vitetta (*sic*; presumably Uhr and Ghetie), Rybak, and Pastan disclose immunotoxin technology which comprises an antibody or antibody fragment that binds to FcγRI as taught by Curnow, Graziano, and Erickson. In addition, the Examiner characterizes Bjerke and Curnow as teaching diseases such as ITP or psoriasis as characterized by an aberrant activity or number of macrophages.

Based on the combination of these eight (8) references, the Examiner concludes that it would have been obvious to one of ordinary skill in the art at the time of the invention to have arrived at the claimed invention. In particular, the Examiner asserts that it would have been obvious to use the monoclonal antibodies (mabs 22, 32, and 197) as taught by Curnow, Graziano, and Erickson in the treatment of human diseases such as those taught by Bjerke and Curnow because these molecules can bind FcγRI without being blocked by endogenous immunoglobulin and because they can be humanized (as taught by Graziano), and combined with any one of the toxins (i.e., Gelonin, Saporin, Exotoxin A, Onconase, and Ricin A) taught by Uhr, Ghetie, Rybak, and Pastan and developed for the treatment of human disease.

Applicants respectfully traverse this rejection. Applicants were the first to recognize that certain diseases, such as skin diseases and other autoimmune diseases, are primarily caused by macrophages, and to develop a method for selectively eliminating or reducing the activity of such macrophages to effectively treat such diseases using e.g., a macrophage-specific immunotoxin. None of the cited references, either alone or in combination, teach or suggest the treatment of macrophage-mediated disorders as claimed by Applicants, let alone in the manner taught by Applicants.

As previously pointed out, Curnow, Graziano, and Erickson teach methods of targeting circulating monocytes or neutrophils – not macrophages as claimed by Applicants.

The references of Uhr, Ghetie, Rybak and Pastan fail to make up for this deficiency, since they pertain only to immunotoxins and teach nothing about the treatment of macrophage-mediated diseases.

In particular, Uhr teaches therapies for targeting B cells (not macrophages) via an epitope unrelated to an Fc receptor. B cells are derived from a lymphoid precursor which

is a completely different hematopoietic lineage from macrophages which are derived from a myeloid precursor. Ghetie is a mere general review discussing how to make antibody/immunotoxins and also fails to teach or suggest the use of the antibody/immunotoxins specifically to treat macrophage-mediated diseases. Similarly, Rybak discloses antibody/immunotoxins (in particular, an RNAse toxin) and fails to teach or suggest the use of such antibody/immunotoxins specifically to treat macrophage-mediated diseases. Pastan also fails to teach or suggest the use of the antibody/immunotoxins to treat macrophage-mediated diseases, as the authors teach only the treatment of prostate cancer cells.

Like the above-discussed references, Bjerke also fails to teach or suggest any method of selectively preventing or treating macrophage-mediated diseases. This reference merely teaches the use Fc binding antibodies to monitor changes in Fc receptor levels in response to therapy – not as a therapy and certainly not to reduce macrophage activity or numbers as claimed by Applicants.

Thus, overall, based on the fact that none of the cited references speak to the prevention or treatment specifically of macrophage-mediated disorders, as claimed by Applicants, it would not have been obvious to have combined the individual teachings of these references in any manner, let alone in the manner claimed by Applicants, to have arrived at the claimed invention. The present rejection is clearly based on hindsight and not on what was taught by the prior art at the time of the invention. Recently, the Federal Circuit noted that "[a] critical step in analyzing the patentability of claims pursuant to section 103(a) is casting the mind back to the time of the invention, to consider the thinking of one of ordinary skill in the art, guided only by the prior art references and the then-accepted wisdom in the field." *In re Werner Kotzab*, 217 F.3d 1365, 1369, 55 USPQ2d 1313 (Fed. Cir. 2000).

Accordingly, based at least on the foregoing, it is respectfully requested that the rejection under 35 U.S.C. § 103 (a) be reconsidered and withdrawn.

Rejection of Claims 1 and 13-17 Under 35 U.S.C. §103 (a)

The Examiner rejects claims 1 and 13-17 under 35 U.S.C. §103(a) as being unpatentable over Curnow, R.T. (cited supra), Graziano et al. (cited supra), Erickson et al (cited supra), in view of McGrath et al. (USPN 5,580,715), Estis et al. (USPN 5,026,557), Rodwell et al. (USPN 4,671,958), Lifson et al. (USPN 4,869,903), and Bagshawe (USPN 5,658,568). In particular, the Examiner states that, in combination, the newly cited references of McGrath, Estis, Rodwell, Lifson, and Bagshawe disclose the targeting of macrophages with a immunotoxin and a liposome using, for example, an antibody or antibody fragment that binds to FcγRI as taught by Curnow, Graziano, and Erickson.

Thus, based on the combination of these eight (8) references, the Examiner concludes that it would have been obvious to one of ordinary skill in the art at the time of the invention to have arrived at the claimed invention.

Applicants respectfully traverse this rejection.

As previously pointed out above, Curnow, Graziano and Erickson fail to teach or suggest any method of preventing or treating macrophage-mediated disorders by selectively targeting macrophages using an Fc receptor binding agent, as claimed by Applicants.

McGrath fails to make up for this deficiency. While the reference refers to the targeting of macrophages using a liposome-based agent, this is carried out using anti-CD-14 and not an agent that binds to an Fc receptor, as claimed by Applicants. In fact, McGrath teaches away from the present invention given that the main cellular expression of CD14 is on monocytes and not macrophages (see, e.g., pg. 398 of *Cellular and Molecular Immunology* by Abbas et al., W.B. Saunders Co. 1991).

Estis merely teaches the preparation of adjuvants comprising liposomes, and thus fails to make up for the previously discussed deficiencies. Moreover, adjuvants are recognized as substances for increasing an immune response and thus would not have been thought applicable to the goal of selectively reducing numers or activity of macrophages to treat particular macrophage-mediated diseases.

Rodwell teaches antibody independent uptake of liposomes in macrophages, and thus also teaches away from any Fc binding agent-mediated method of selectively killing macrophages, such as that claimed by Applicants. While the reference states that liposomes are readily phagocytosed by macrophages, it also teaches that this is dependent on "[w]hether or not the liposomes are coated with antibody molecules" (Col. 2, lines 35-38). Accordingly, the reference would not have been considered relevant to the gola of Fc receptor mediated macrophage-specific cellular killing.

Lifson teaches the targeting of a protein using a carrier anti-macrophage antibody, but solely to prevent HIV replication or infection and not to reduce the activity or number of macrophages as claimed by Applicants. Moreover, the reference neither teaches nor suggests targeting a macrophage using an Fc receptor binding agent.

Bagshawe also fails to render the claimed invention obvious in any way since it does not teach or suggest targeting of macrophages whatsoever, much less using an Fc binding agent of the claimed invention.

Overall, the Examiner has simply combined both here and in the foregoing §103(a) rejection, using hindsight, references which at best teach a general component used in the novel and inventive method of the invention. The Examiner has ***not*** provided evidence of the requisite motivation for why, at the time of the invention (i.e., ***without*** the

benefit of hindsight) one of ordinary skill would have been motivated to have combined these references in the manner suggested by the Examiner). Indeed, the mere fact that the prior art could be modified does not make the modification obvious unless the prior art suggested the desirability of the modification. *In re Laskowski*, 871 F.2d 115, 117, 10 USPQ2d 1397, 1398 (Fed. Cir. 1989).

Overall, based on the above, the cited combination of references fails would not have rendered obvious at the time of the present invention a method of selectively reducing the number or activity of macrophages (e.g., to treat a disorder characterized by aberrant numbers or activity of macrophages) using an agent which specifically binds to macrophages via an Fc receptor and an agent which kills or reduces the activity of the macrophages, as claimed by Applicants. Again, the rejection is clearly based on hindsight and not on what was taught by the prior art at the time of the invention. *In re Werner Kotzab*, 217 F.3d 1365, 1369, 55 USPQ2d 1313 (Fed. Cir. 2000).

Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. §103(a), be withdrawn.

CONCLUSION

In view of the foregoing, entry of the amendments and remarks herein, reconsideration and withdrawal of all rejections, and allowance of the instant application with all pending claims are respectfully solicited. If a telephone conversation with Applicants' attorney would help expedite the prosecution of the above-identified application, the Examiner is urged to call Applicants' attorney at (617) 227-7400.

Respectfully submitted,
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Attachment: Appendices A-B

Appendix A

1. A method of selectively reducing the number or activity of macrophages, comprising contacting the macrophages with a macrophage-binding compound comprising (a) an agent which binds to an Fc receptor at a site which is distinct from that bound by endogenous immunoglobulins to; and (b) an agent which kills or reduces the activity of the macrophages.

2. A method of treating or preventing a disease in a subject characterized by aberrant activity or number of macrophages within a selected area of the subject, comprising locally administering to the area a macrophage-binding compound comprising (a) an agent which binds to an Fc receptor; and (b) an agent which kills or reduces the activity of the macrophages.

3. The method of either of claims 1 or 2, wherein the agent which binds to an Fc receptor binds at a site which is not bound by an endogenous immunoglobulin.

4. The method of either of claims 1 or 2, wherein the Fc receptor is an Fcγ receptor (FcγR) or an Fcα receptor (FcαR).

5. The method of claim 4, wherein the Fcγ receptor is selected from the group consisting of FcγRI, FcγRII and FcγRIII.

6. The method of claim 5, wherein the Fcγ receptor is a human FcγRI.

8. The method of either of claims 1 or 2, wherein the macrophage-binding compound comprises an anti-Fc receptor antibody conjugated to a toxin.

9. The method of claim 8, wherein the anti-Fc receptor antibody is an anti-Fc γ receptor antibody or a fragment thereof.

10. The method of claim 9, wherein the anti-Fcγ receptor antibody is a monoclonal antibody selected from the group consisting of mab 22, 32 and 197, or a fragment thereof.

11. The method of claim 9, wherein the anti-Fcγ receptor antibody is a humanized antibody H22 produced by the cell line having ATCC accession number CRL 1117 or a fragment thereof.

12. The method of claim 8, wherein the toxin is selected from the group consisting of Gelonin, Saporin, Exotoxin A, Onconase and Ricin A.

13. The method of claim 1, wherein the agent which kills or reduces the activity of the macrophages is encapsulated within a liposome.

14. The method of claim 13, wherein the agent which kills or reduces the activity of a macrophage is dichoromethylene diphosphonate (CL2MDP) or derivatives thereof.

15. The method of claim 13, wherein the agent which binds to an Fc receptor is a single chain antibody.

16. The method of claim 13, wherein the agent which binds to an Fc receptor is an anti-Fcγ receptor antibody or a fragment thereof.

17. The method of claim 13, wherein the agent which binds to an Fc receptor is a single chain anti-Fcγ receptor antibody or a fragment thereof.

18. The method of claim 1, wherein the contacting step occurs in culture.

19. The method of either of claims 1 or 2, wherein the macrophage-binding compound is administered topically, intradermally or subcutaneously in a pharmaceutically acceptable carrier.

20. The method of claim 2, wherein the disease is characterized by enhanced proliferation and/or growth factor secretion of the macrophage.

21. The method of claim 2, wherein the disease is selected from the group consisting of psoriasis, atopic dermatitis, scleroderma, cutaneous lupus erythematosis, Human Immunodeficiency Virus infection, multiple sclerosis, rheumatoid arthritis, Chronic Polymorphic Light Dermatosis, Chronic Obstructive Pulmonary Diseases, and Wegener's Granulomatosis.

Appendix B

CRYOBIOLOGY 24, 91–102 (1987)

Selective Destruction of Leucocytes by Freezing as a Potential Means of Modulating Tissue Immunogenicity: Membrane Integrity of Lymphocytes and Macrophages[1]

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It is now known, when a tissue allograft is transplanted, that antigen recognition alone is not sufficient for lymphocyte activation in the host. "Passenger" leucocytes (antigen-presenting cells) present in the donor tissue are now recognized as a major immunogenic stimulus. Removal of these contaminating leucocytes, using a variety of procedures, has enabled the immunogenicity of allografts to be reduced, thus enhancing the survival of tissue allografts. This initial study explores the possibility of using a cryobiological approach to modulating the immunogenicity of tissues by virtue of the well-recognized differential susceptibility of different cell types to freezing injury. The investigation was prompted by demonstrations that pancreatic islets can secrete insulin in response to a graded glucose challenge after cryopreservation using relatively fast cooling rates which would be expected to be suboptimal for leucocyte survival. Batches of rat peripheral blood lymphocytes, or peritoneal exudate cells (macrophages) were cooled at 0.3, 1, 5, 20, 75, or 200°C/min using three different cryopreservation protocols reported to yield viable pancreatic islets. Cell survival was evaluated in terms of the numbers of cells recovered after freezing as well as a fluorometric viability assay which assessed the membrane integrity of cells. Optimum survival of both lymphocytes and macrophages after freezing and thawing was found at cooling rates in the range of 0.3 to 5°C/min. A significant number (10–40%) of these lymphoid cells survived freezing at 20°C/min and only after cooling at rates greater than 75°C/min was survival reduced to a negligible level.

INTRODUCTION

Reducing the immunogenicity of tissue allografts prior to transplantation would facilitate greater acceptance of the graft, even in the absence of immunosuppression (16, 28). Until recently the immunological rejection of grafted tissues was believed to be induced by the response of the host's lymphocytes to histocompatibility antigens that were expressed on all parenchymal cells. It is now recognized, however, that antigen recognition alone is not sufficient for lymphocyte activation. Apparently "passenger" leucocytes (antigen-presenting cells) carried in the donor tissue provide the major immunogenic stimulus for the host (19). A variety of techniques have been used to deplete donor tissues of contaminating leucocytes, thereby reducing immunogenicity which results in a marked prolongation of tissue allografts (19, 28), including thyroid (18), parathyroid (25), pancreatic islets of Langerhans (16, 17), kidney (20), and heart (21). The basic principle is one of selective cytotoxicity, to achieve destruction of allostimulatory leucocytes while preserving the function of the graft.

In this initial study we have begun to explore the possibility of using the differential susceptibility of distinct cell types to freezing injury (22, 35) to modulate the immuno-

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genicity of tissues. The differential sensitivity of various cell types to the conditions of cryopreservation, particularly the cooling rate, has been used to select for various cell types (see 8, 28). Our approach is to find specific conditions of freezing and thawing which are known to favor the survival of parenchymal cells of a graft while selectively killing the immunocompetent passenger leucocytes.

Advances have been made in recent years in ameliorating diabetes by transplanting pancreatic islets (17). In addition substantial progress has been made in developing methods of cryopreservation for islet tissue (see 4, 35). This study was prompted by reports that isolated rat islets of Langerhans can secrete insulin in response to a glucose challenge after cryopreservation using relatively fast cooling rates (4, 5, 37), and normoglycemia has been restored in diabetic mice (31) and dogs (39) using pancreatic endocrine tissue cryopreserved using fast cooling rates. Such rates would be expected to exceed the optimal cooling rate for leucocyte survival (15).

Transplantation must ultimately be used to determine if the immunogenicity of tissue allografts is altered by freezing and thawing. However, it is not practical to use such expensive and time-consuming procedures to examine the large number of permutations of cryobiological conditions to establish the most effective method for selectively killing passenger lymphoid cells. In addition such *in vivo* assays do not have the sensitivity to distinguish between the efficacy of relatively minor changes in procedures. The *in vitro* study described here and briefly elsewhere (36) was undertaken, therefore, to examine in detail the effect of cooling rate on the survival of rat lymphocytes and macrophages after freezing and thawing by procedures which have been reported to be effective in cryopreserving isolated islets of Langerhans (5, 29, 37). In this initial investigation cell survival was evaluated by supravital staining using the fluorescent markers acridine orange and propidium iodide (AO/PI). Fluorometric assays for membrane integrity are simple and quick and compared with other tests such as trypan blue exclusion are more stable, easier to read, and subject to fewer staining artifacts (14, 27). Furthermore, this AO/PI test is ideally suited to assaying the large number of samples in this study and is applicable to testing the viability of both types of mononuclear cells (lymphocytes and macrophages).

METHODS AND MATERIALS

Preparation of cell suspensions. Purified suspensions of either lymphocytes or macrophages were prepared by standard procedures from Long–Evans Hooded rats (Charles River, NJ) weighing 200–300 g. Animals were anaesthetized with diethylether. Lymphocytes were prepared aseptically from peripheral blood obtained by cardiac puncture (18-gauge needle) using citrate–phosphate–dextrose (CPD) as the anticoagulant (sodium citrate · $2H_2O$, 26.3 g/liter; citric acid, 3.27 g/liter; monobasic sodium phosphate · H_2O, 2.20 g/liter; dextrose, 25.5 g/liter). Blood was drawn into a plastic syringe containing 0.9 ml CPD per 5 ml of blood.

Erythrocytes were sedimented by mixing 5 vol of blood with 1 vol of a 6% solution of dextran (T500, Pharmacia Fine Chemicals, Uppsala, Sweden) in Hank's balanced salt solution without calcium or magnesium (HBSS(−); Gibco, Grand Island, NY) containing 25 m*M* Hepes (Sigma, St. Louis, MO) (6). Sedimentation of erythrocytes was allowed to take place at room temperature for 20–30 min in a plastic syringe (50 ml) standing upright on its plunger. The leucocyte-rich supernatant was then expressed into a polypropylene tube through a butterfly infusion set with the needle removed. The plasma was diluted with cold HBSS(−) and leucocytes were sedimented by centrifugation at 4°C (200*g*, 10 min). The

platelet-rich supernatant was then removed by aspiration before the leucocyte pellet was resuspended in HBSS(−) and layered gently onto the surface of a "lymphocyte separation medium" (6.2% (w/v) Ficoll + 9.4% (w/v) sodium diatrizoate; density 1.078 g/ml at 20°C: Litton Bionetics, Inc., Charleston, SC). After centrifugation (400*g* for 20 min at 4°C), mononuclear cells were harvested from the interface of the discontinuous density gradient and washed twice in HBSS(−). Finally, the purified cell suspension was resuspended in tissue culture medium (RPMI 1640 + 20 m*M* Hepes + 10% serum; Gibco) and samples were removed for cell counting in either a Coulter counter or hemacytometer. Differential cell counts were performed to determine the purity of the lymphocyte preparation using cytocentrifuge preparations after Romanowsky staining ('DIFF-QUIK' stain, Dade Diagnostics, Inc., Aguada, PR).

Suspensions of "resident" macrophages were prepared from nonelicited peritoneal exudates obtained by injecting 15 ml HBSS(−) intraperitoneally and massaging the abdomen for 2 min. Peritoneal fluid was then withdrawn and exudate cells were purified on a discontinuous density gradient in the same way as described above for peripheral blood lymphocytes. Cell counting and quantitation of the different cell populations are described above.

Experimental design. The recovery of both lymphocytes and macrophages was assessed after freezing and thawing by one of four procedures. Batches of lymphocytes or macrophages suspended in RPMI 1640 medium with 10% serum were cooled at one of six rates (0.3, 1, 5, 20, 75, or >200°C/min) using four different cryopreservation procedures with dimethyl sulphoxide (Me_2SO) as the cryoprotectant; three procedures were those reported to yield viable pancreatic islets of Langerhans after freezing and thawing and the fourth, which involved cooling at 1°C/min only, was a control procedure for optimum survival of lymphocytes and macrophages after storage in liquid nitrogen.

Procedures for freezing and thawing. The procedures used in this study for freezing and thawing are summarized schematically in Figs. 1 and 2. In all procedures cell suspensions were dispensed at known concentrations between 1×10^6 and 1×10^7 cells/ml and handled throughout in polypropylene freezing ampoules with screw caps (5 ml; NUNC, Denmark). The control method of cryopreservation for these mononuclear cells involved a gradual introduction of 10% (w/v) Me_2SO to the cell suspensions at 0°C, and controlled cooling at 1°C/min to −60°C. Samples were thawed quickly from storage at −196°C and the cryoprotectant was removed by serial dilution (four steps at 5-min intervals in which the concentration of Me_2SO was halved each time) at 0°C (7, 9, 26, 41).

Islet cryopreservation protocol 1 (ICP1) followed the method of Rajotte *et al.* (29) in which cells were fully equilibrated with 1 mol/liter of Me_2SO during a 30-min exposure at 22°C followed by the addition of up to 2 *M* Me_2SO at 0°C before the onset of freezing and controlled rate cooling. After storage in liquid nitrogen at −196°C, samples were thawed slowly (10°C/min) and the cryoprotectant was removed from the cells using a sucrose dilution technique at 0°C to minimize osmotic cell swelling during dilution. Finally, the sucrose was diluted gradually over 15 min at 22°C and the cells were resuspended in isotonic medium.

Islet cryopreservation protocol 2 (ICP2), which was the method of Bank *et al.* (5), involved a relatively short exposure (6 min) to cryoprotectant (1 *M* Me_2SO) at 0°C prior to freezing and controlled cooling. Samples were thawed rapidly from −196°C by agitation in a water bath at 37°C and the cryoprotectant was diluted stepwise at 37°C by adding doubling volumes of HBSS + 10% serum at 5-min intervals. The third islet cryopreservation protocol (ICP3) was the same as ICP1 except samples were thawed

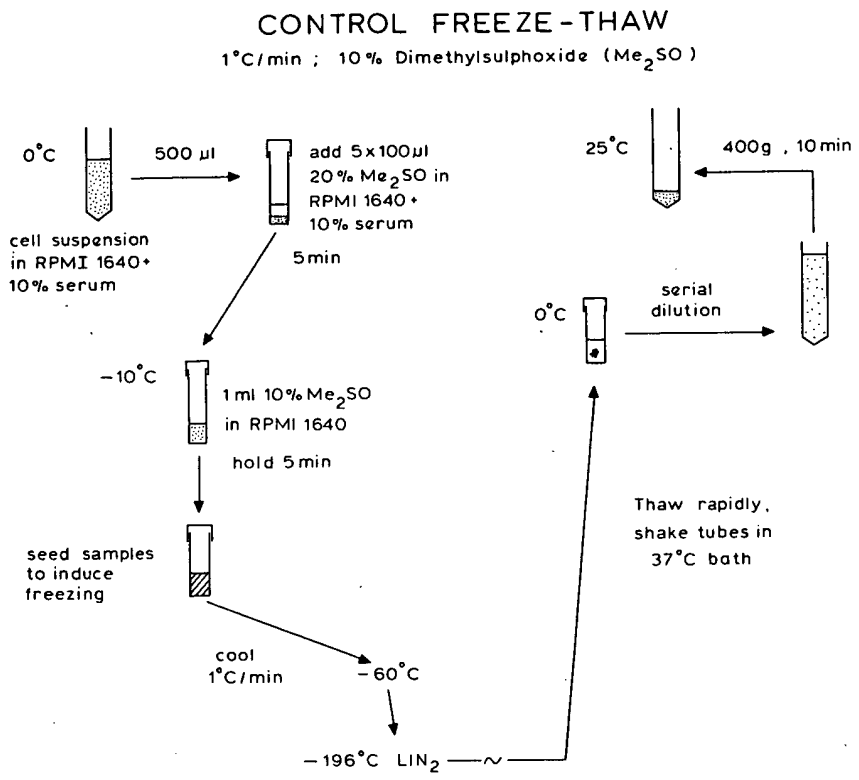


FIG. 1. Schematic diagram outlining the control procedure used for freezing and thawing leucocytes by an optimized protocol.

rapidly instead of slowly since Taylor and Benton (37) had previously found that pancreatic islets frozen by this procedure yielded highest survival when the samples were thawed quickly (50°C/min).

In all experiments cells were resuspended in a known volume of isotonic culture medium (RPMI 1640 + 10% serum) after thawing and the concentration of recovered cells was redetermined using the same procedures described above. Samples were incubated at 37°C for 3 hr before testing for viability. In each experiment it was necessary to hold some samples for an additional time not exceeding 2 hr which represented the total time necessary to process all samples of a single experiment in the AO/PI assay. In the five replicate experiments conducted for each set of conditions, individual samples were assayed in a random sequence in order to minimize the unlikely event that the short additional holding periods at 37°C incurred any extra benefit or detriment to cell survival.

Assessment of cell viability. Cell survival after freezing and thawing was evaluated as the percentage of cells recovered as well as by supravital staining for membrane integrity of the recovered cells using, in combination, the fluorescent probes acridine orange (AO) and propidium iodide (PI) (24, 42). This assay differentiates between viable and nonviable cells by the simultaneous use of inclusion and exclusion dyes.

Acridine orange is a membrane-permeable fluorescent dye. At low concentrations it exists as a monovalent cation that binds to nucleic acids and causes DNA to fluoresce green and red-orange cytoplasmic fluorescence. The appearance of cells

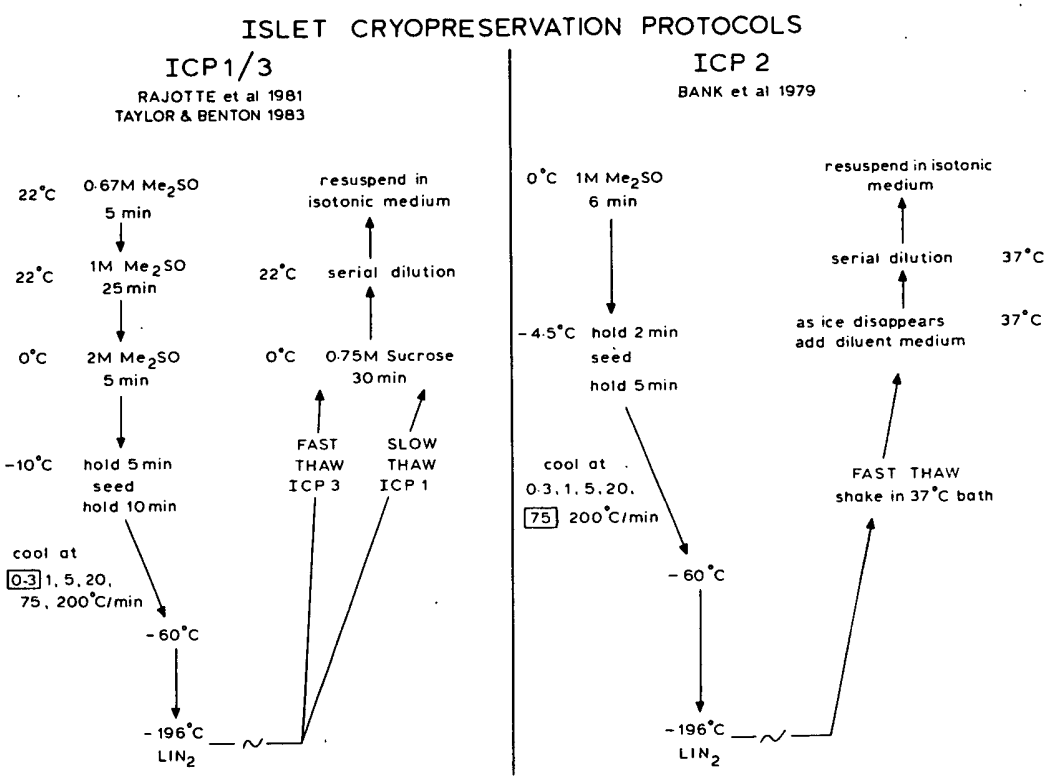


FIG. 2. Flow diagram of the three islet cryopreservation protocols used in this study to examine the survival of lymphocytes and macrophages after cooling at one of six cooling rates. As described in the text these procedures were based on previously published methods: ICP1 (Rajotte *et al.* (29)), ICP2 (Bank *et al.* (5)) and ICP3 (Taylor and Benton (37)). ICP1 was originally published for cooling islets of Langerhans at 0.3°C/min and ICP2 for cooling islets at 75°C/min as indicated by the boxed cooling rates. In this study additional cooling rates were used as indicated.

stained with AO/PI is comparable to cells stained with fluorescein diacetate and ethidium bromide but AO has the advantage that the pattern of fluorescence reveals nuclear morphology, thereby allowing identification and exclusion of "contaminating" leucocytes (eosinophils and polymorphonuclear neutrophils) from the count of cells within a particular preparation. Propidium iodide is an analog of ethidium bromide but has a higher quantum yield and is more resistant to photobleaching than ethidium bromide. It is an intercalating dye which is excluded from intact cells and permeates only through the membranes of dead or dying cells (42, 44); propidium iodide excludes AO competitively from the nuclei of damaged cells and fluoresces red under uv illumination (13, 34).

A sample of the cell suspension was mixed on a microscope slide with an equal volume of 20 μmol/liter acridine orange and 20 μg/ml propidium iodide in Hank's balanced salt solution (final concentration 10 μmol/liter AO and 10 μg/ml PI). Samples were incubated at room temperature for 10 min before examination under an inverted microscope (WILD M40) equipped for epi-illumination with a Zeiss Xenon light source and an FITC filter module (490-nm excitation filter and 510-nm barrier filter). A minimum of 500 fluorescent cells were counted for each assessment and viability

was expressed as the ratio of green nuclei to the total of green + red nuclei.

RESULTS

For each experiment the survival data has been expressed by three separate indices: The recovery of cells was calculated as a percentage of the initial numbers of cells prior to freezing (Table 1). The membrane integrity of cells recovered after freezing and thawing was determined by the AO/PI fluorescence assay. In addition the mean percentage viable yields were calculated by expressing the number of viable cells as a percentage of the number of cells present prior to freezing. The "survival index" therefore reflects the loss of cells, a parameter often ignored in cryopreservation studies, as well as the change in membrane integrity due to freezing and thawing. Membrane integrity and viable yield curves for cells frozen and thawed by all procedures are shown for comparison in Fig. 3.

Recovery of Cells

Cell recoveries after the complete freezing and thawing process, including the removal of cryoprotectant by dilution, are shown in Table 1. The yield of macrophages after cooling at any rate in the three islet cryopreservation protocols ranged between 60 and 70%. In some cases recovery was significantly lower than the yield of macrophages after freezing by the optimized control procedure where cooling was 1°C/min (Table 1). Post-thaw recovery of macrophages frozen by the ICP2 procedure was significantly lower at all cooling rates than the recovery obtained using the control procedure.

For lymphocytes, post-thaw recoveries were comparable, for the three islet cryopreservation protocols, at cooling rates <5°C/min (56–72%). However, recovery diminished with increasing cooling rates above 5°C/min.

Recovery of Viable Cells

Figure 3 shows that the recovery of both lymphocytes and macrophages and their membrane integrity is markedly dependent upon the rate of cooling in each of the cryopreservation procedures. Optimum recovery of both cell types was achieved using cooling rates in the range of 0.3 to 5°C/min. Similar recovery of cells was observed after cooling at 1°C/min by the standard control procedures for leucocytes, thus corroborating previous studies (7, 9, 15, 26, and 41). The general shape of the cooling rate versus survival curve was similar for both lymphocytes and macrophages for all three protocols tested. The viable yield of lymphocytes and macrophages using the ICP1 procedure falls precipitously at cooling rates >10°C/min and negligible recovery of cells occurred at rates >50°C/min. ICP1 involves slow warming which would favor recrystallization of intracellular ice that is likely to have formed at faster cooling rates. The combination of faster warming with cooling rates >10°C/min (ICP2 and ICP3) resulted in higher recoveries at the faster rates. The optimum cooling rates for survival were the same but it was noticeable that using the ICP2 and ICP3 procedures a higher proportion of both lymphocytes and macrophages survive cooling at faster rates with significant recoveries at 20°C/min and 10–20% survival at rates as high as 75°C/min.

Statistical Analysis

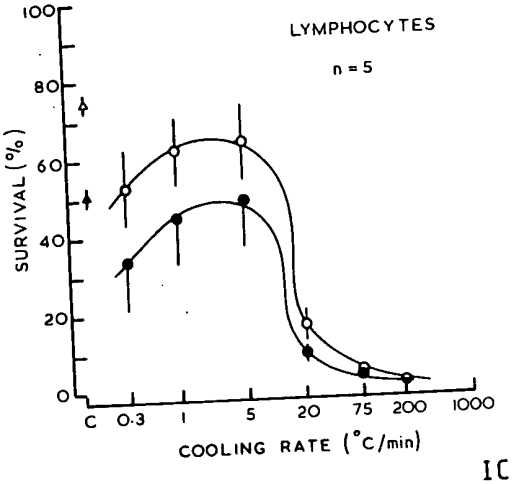
A three-factor analysis of variance (ANOVAR) was applied to the data for viable yields (32). A number of conclusions can be drawn from this analysis which is summarized in Table 2. The effect of both cooling rate and the method of cryopreservation are highly significant ($P < 0.001$), but the type of cell (lymphocyte and macrophage) is not. The analysis also shows that there are no significant interactions be-

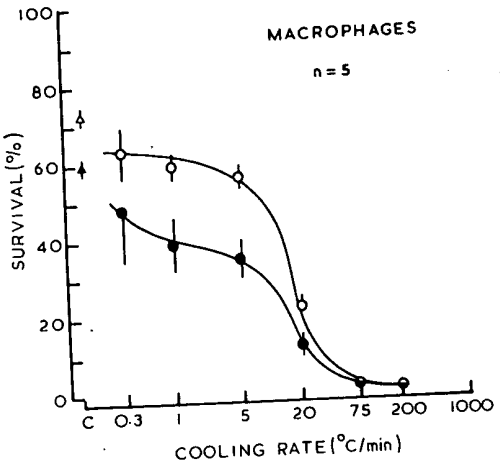
TABLE 1
Recovery of Cells after Freezing and Thawing[a]

Freeze/thaw protocol	Cell type	Cooling rate (°C/min) 0.3	1	5	20	75	200	n
Control	Lymphocyte	—	67 ± 3	—	—	—	—	10
	Macrophage	—	83 ± 4	—	—	—	—	10
ICP1	Lymphocyte	71 ± 17	68 ± 10	72 ± 9	64 ± 7	54 ± 9	50 ± 8*	5
	Macrophage	72 ± 17	66 ± 10	63 ± 9**	53 ± 9**	70 ± 14	66 ± 8	5
ICP2	Lymphocyte	61 ± 3	56 ± 5	60 ± 5	47 ± 6**	27 ± 6**	19 ± 2**	5
	Macrophage	58 ± 4**	63 ± 5*	59 ± 4*	57 ± 4**	61 ± 6**	53 ± 7**	5
ICP3	Lymphocyte	70 ± 8	73 ± 7	72 ± 6	73 ± 6	64 ± 6	57 ± 7	5
	Macrophage	68 ± 10	67 ± 7	70 ± 10	73 ± 11	71 ± 7	71 ± 4	5

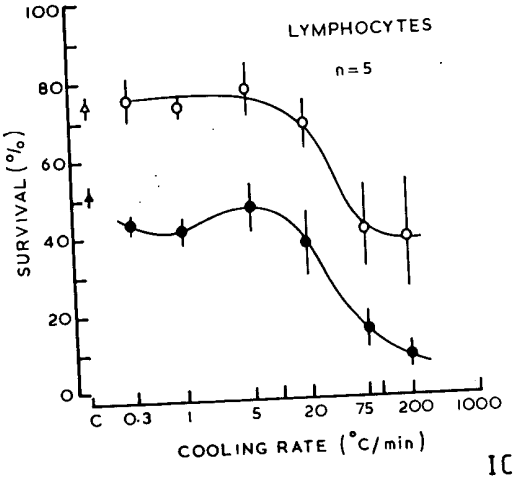
[a] Percentage recovery of cells relative to the known numbers of cells before freezing.
* Value significantly different ($P < 0.05$) from the recovery obtained using the control optimized procedure for freezing and thawing.
** Values significantly different from control values ($P < 0.01$).

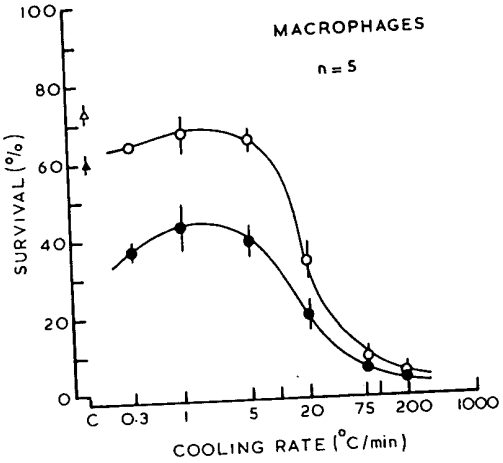
ICP1



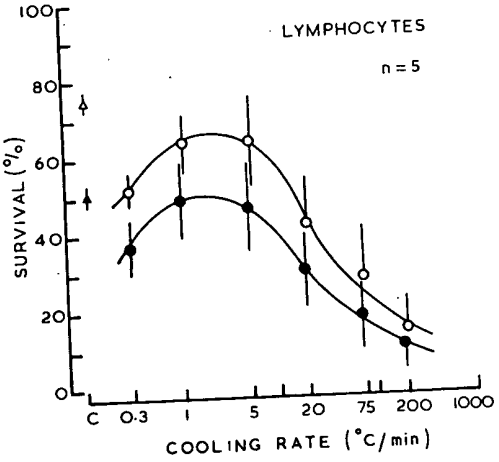


ICP2





ICP3



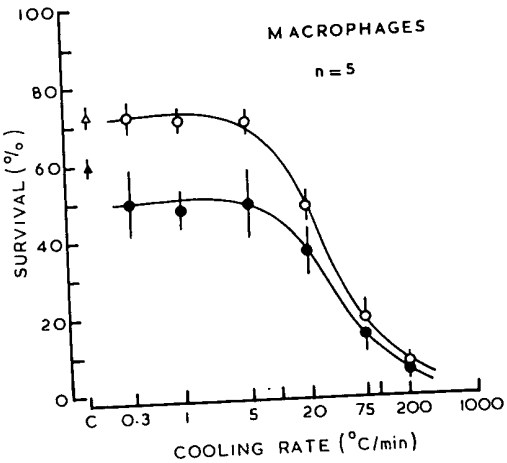


TABLE 2
Analysis of Variance on Data for Viable Yields

Factor	Degrees of freedom	Mean squares	Variance ratio (F)	Probability (P)
Cryopreservation protocol (ICP)	2	1523	7.719	9.75×10^{-4}
Cooling rate (CR)	5	10228	51.838	8.06×10^{-11}
Cell type (CT)	1	195.8	0.992	0.678
First-order interactions				
ICP × CR	10	189.8	0.962	0.520
ICP × CT	2	356	1.804	0.1662
CR × CT	5	152.3	0.772	0.573
Second-order interactions				
ICP × CR × CT	10	112.3	0.569	0.837
Residual	144	197.3	1	

tween these factors: for example, the marked effect of cooling rate is independent of the protocol or type of cell frozen.

DISCUSSION

Since the first reports in 1962 (3), the cryobiology of leucocytes has been studied extensively (see 15). However, most studies relate to the cryopreservation of lymphocytes using slow cooling rates of the order of 1°C/min since this is clearly an optimum rate for high survival. Few studies have examined the effect of cooling rate varied over several orders of magnitude and in the case of macrophages, investigation of cooling rate sensitivity appears to be limited to the single study by Foreman *et al.* on murine peritoneal exudate cells (9). Mononuclear phagocytes are now known to be essential components in the regulatory role of immune responses (30), and their antigen-presenting function is particularly important in the context of tissue immunogenicity (40). This study, therefore, contributes to a more complete understanding of the cryobiology of mononuclear leucocytes by comparing, for the first time, the cooling rate sensitivity of both lymphocytes and macrophages obtained from a single species (the rat), frozen and thawed by identical procedures and assayed in the same way.

The survival of lymphocytes and macrophages was found to be highly dependent upon cooling rate when frozen and thawed by any of the islet cryopreservation protocols. Each protocol differs in the variables known to interact and influence the survival of cells; these include the concentration of cryoprotectant (CPA), length and temperature of exposure to CPA, the dilution regimen, and the cooling rate used. Al-

FIG. 3. Panel of "survival curves" for lymphocytes and macrophages as a function of cooling rate using three different cryopreservation protocols. The viability indices of cells recovered after freezing and thawing determined by the AO/PI assay for membrane integrity are shown by open symbols (○). Values are the means (±SEM, $n = 5$). The mean (±SEM) percentage viable yields calculated by expressing the number of viable cells as a ratio of the number of cells present prior to freezing are shown by closed symbols (●) ($n = 5$). Survival indices for cells frozen and thawed by the control method of cryopreservation are also shown: (△) membrane integrity index, (▲) mean percentage viable yield index ($n = 10$).

though the sensitivity of these leucocytes to cooling rate was expected, it was not previously known precisely what level of recovery would be attained at different rates using these various procedures. Attempts to modulate the immunogenicity of islets during cryopreservation will require the destruction of most, if not all of the immunocompetent passenger leucocytes. The design of procedures to achieve this will, therefore, require an estimate of the cryopreservation parameters most likely to result in the total destruction of leucocytes under conditions known to preserve the viability of the tissue. Furthermore, information on the relative recoveries of leucocytes using these different cryopreservation procedures will be valuable in selecting conditions for the immunohistochemical studies *in vitro* and transplantation studies *in vivo* needed to investigate more completely the possibility of using this approach to prolong the survival of allografts.

A proportion of cells are lost during these cryopreservation procedures. It is uncertain whether most of these cells are lost during freezing and/or after the cells are thawed. In some samples resuspension of frozen/thawed cells was difficult, indicating that clumping of cells may partly account for reduced recoveries of cells. The magnitude by which each cell population was depleted is similar to that reported by other investigators for both lymphocytes (2, 11, 26, 33, 38, 43) and macrophages (1, 12, 41).

The survival curves shown in Fig. 3 for both lymphocytes and macrophages resemble the classic profiles established by studies on a wide variety of cell types. Mazur has convincingly explained such curves in terms of a two-factor hypothesis of freezing injury; each factor is oppositely dependent on cooling rate and maximum cell recovery after freezing is obtained at cooling rates fast enough to minimize "solution effects," but slow enough to avoid intracellular freezing (22, 23, 35). On the basis of our fluorescent AO/PI membrane integrity assay cooling rates of approximately 100°C/min are required to reduce the viable yield of leucocytes to negligible levels after freezing by these islet cryopreservation procedures.

Although assessment of membrane integrity is ideal for screening the large number of variables examined in this study, it gives no indication of the immunocompetency of the surviving cells. In fact, several reports show that membrane integrity tests may indicate a higher viability than do assays of active functions (7, 10, 15, 38). If this applies also to the function of antigen-presenting cells then the degree of survival at any cooling rate would be lower than that given by the survival curves in Fig. 3 and total depletion of allostimulatory leucocytes would be expected at cooling rates in the range of 75–100°C/min. Additional studies are currently in progress to correlate membrane integrity with the recovery of cell functions; some preliminary data have been published (36).

These experiments show that at fast cooling rates the ICP1 protocol coupled with slow warming causes the most efficient destruction of leucocytes and that the viable yield of both lymphocytes and macrophages was negligible after cooling at 75°C/min. These conditions would promote cell destruction by favoring the recrystallization of intracellular ice formed at these cooling rates (see 22). Cell recovery at these faster rates was higher using ICP2 and ICP3 which both involved faster rates of warming. A two-factor analysis of variance (32) for the viable yields of both lymphocytes and macrophages after cooling at 75°C/min shows that the effect of the different protocols is significant at the 95% level of confidence. The analysis also supports the conclusion that the effect is independent of cell type.

The similar responses shown by peripheral blood lymphocytes and peritoneal

macrophages to freezing and thawing suggest that similar behaviour would be expected from dendritic leucocytes. The dendritic cell is similar in many ways to the tissue macrophage and although the cryobiological properties of dendritic cells have not yet been determined specifically, it is likely that they would be similar to the leucocytes studied here.

We have recently reported on the interaction of cooling rate, warming rate, and the extent of permeation of cryoprotectant on the survival of cryopreserved isolated islets of Langerhans (37). It was shown that the effects of warming rate and the extent of permeation of cryoprotectant are highly significant although the effect of cooling rate was not. It is envisaged, therefore, that conditions of freezing and thawing can be selected to provide adequate survival of the endocrine tissue and almost total ablation of lymphoid cells. This study defines clearly the conditions most likely to effect a depletion of "passenger" lymphoid cells by freezing during the cryopreservation of donor tissue.

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